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The horseradish peroxidase technique applied to the teleostean nervous system

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Experimental neuroanatomical research of fish material by the degeneration technique often is seriously hampered by the unpredictable reaction of degenerated nervous structures to 'standard' silver stains such as the Nauta–Gygax and the Fink–Heimer methods. Consequently, numerous trial experiments are required to adapt such variables as survival time and temperature of the survival tanks to the requirements of the species and the type of fiber system under study[10]. Furthermore, in a study of secondary sensory connections of the respiratory system within the brain stem of the carp (*Cyprinus carpio* L.) by the degeneration technique, we found that even small electrolytic lesions were often lethal. Moreover, the repeated penetration of the brain with silver microelectrodes for electrophysiological determination of the lesion site generally caused considerable non-specified fiber degeneration (see experimental procedure 5 in Table I).

It is clear from the foregoing that an alternative to the degeneration technique for studies of fiber connections of both peripheral and central nervous structures was urgently needed. Recent reports on the use of the enzyme horseradish peroxidase (HRP) as a neuroanatomical tracer substance in higher vertebrates therefore appeared quite promising. In addition to other useful features this method has been reported to allow both anterograde and retrograde axonal transports[5–9,11,12]. The results reported here show that the HRP technique is indeed very suitable for the tracing of fiber connections in the teleostean nervous system.

The experiments in this study were performed in 26 specimens of the species *Cyprinus carpio* L., measuring about 30 cm in length. A survey of the experimental groups is listed below. Note that in several cases two experiments were carried out in a single animal. This was justified for the reason that a number of initial experiments had failed to reveal any contralateral distribution of primary sensory or motor fibers.

The animals were perfused with teleost saline solution containing 8 I.U. heparin per ml followed by a solution of 2–4% formaldehyde and 2–5% glutaraldehyde in 0.1 *M* phosphate buffer (pH=7.4)[4]. Brains including ganglia were dissected out, embedded in 20% gelatin, postfixed overnight in fresh fixative and rinsed for 24 h in

TABLE I

Throughout the experimental period the temperature of the survival tanks was kept at about 16–17 °C.

Experimental procedure	*No. of experiments*	*Survival time (days)*
(1) Intramuscular injection of 30 μl HRP (type VI Sigma) 5% in saline in the levator hyomandibulae or adductor mandibulae.	15	2–12
(2) Microinjection* of 3–10 μl HRP 5% in branches of NV and NVII between their innervation sites and the central nervous system.	10	4–11
(3) Microinjection* of 1.5–3.0 μl HRP 5% over a time period of 10 min in the cerebellum.	2	2–3
(4) Electrophoretic** deposit of HRP in the cerebellum.	4	7–10
(5) Electrophoretic*** deposit of HRP in the rostral trigeminal motor nucleus (nMV) following recording of cellular activity for identification of the injection site.	2	5–6

* In these cases glass micropipettes with tip diameters of 20 μm were used, connected with a 5 ml syringe driven by an infusion pump.

** Electrophoresis was applied by means of glass micropipettes, tip diameter 10 μm, filled with 5% HRP in Tris–HCl buffer (pH=8.2). Current=1–2 μA, DC positive at 1 sec intervals on–off. Total on-time: 10 min[3].

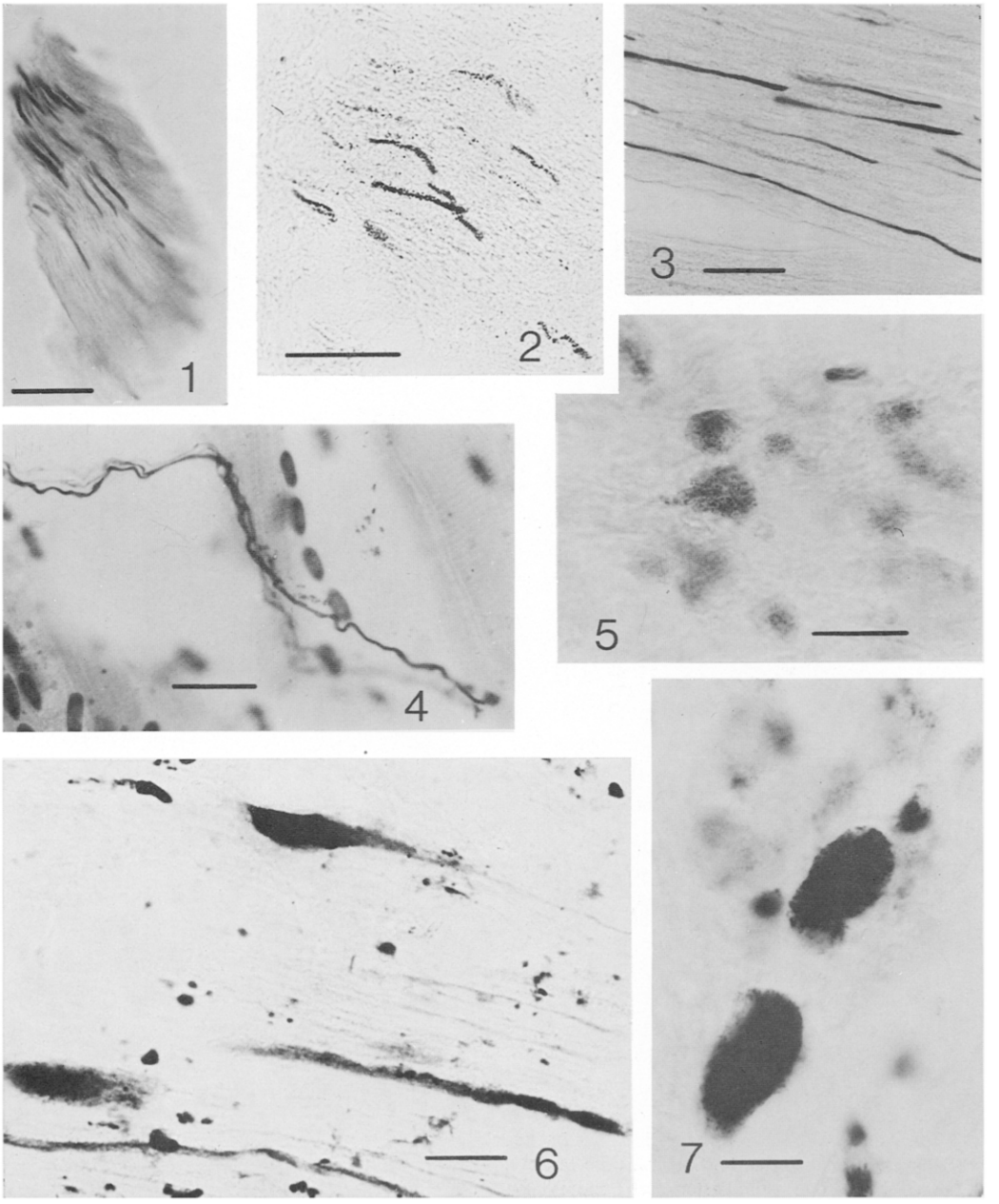
*** Here the same type of glass micropipettes were used as in the above mentioned cases. Electrical activity was recorded from cells firing in respiratory rhythm (Fig. 14a). The functional character of the cells was determined by cross-correlation with electromyograms of the respiratory muscles[1].

0.1 *M* phosphate buffer (pH=7.4), containing 5% sucrose. The blocks were sectioned on a freezing microtome at 40 μm, rinsed in 0.05 *M* Tris–HCl buffer (pH=7.5), immersed for 10 min in buffer of the same composition but supplemented with 0.05% diaminobenzidine tetrahydrochloride and 0.01% H_2O_2, then rinsed in distilled water, dehydrated and mounted[2]. In some cases adjacent sections were stained according to the procedure described by Lynch *et al.*[11], using benzidine dihydrochloride as a substrate for the enzyme.

The same procedures were applied to muscle tissue of some specimens in experimental group 1, and to nerves distal to the injection site, as well as to the innervated muscles in some cases of experimental group 2 (Table I).

Some observations on Table I. In *intramuscular injection* (experimental group 1), labeling of the motor cell bodies in several parts of the motor trigeminal nucleus by the enzyme could not be detected for at least 10 days after intramuscular HRP injection (Figs. 9 and 10).

When using *nerve injections* (experimental group 2), HRP injected into the trigeminal nerve intracranially resulted in axonal uptake and retrograde transport over 10 mm in a 4-day survival time. Ganglion cell bodies close to the injection site were also found HRP-positive (Fig. 6). More peripheral injections, about 20 mm from the brain stem, caused labeling of motor fibers within the CNS after 6 days survival (Figs. 1 and 2). In addition, weak labeling of ganglion cells located 15 mm proximal from the injection site was observed. In cases of survival times of up to



Figs. 1 and 2. Labeled fibers of the trigeminal nerve at their entrance into the brain stem after injection of HRP in a peripheral nerve at 20 mm distance. Survival time 6 days. Fig. 1: stained according to Karnovsky; Fig. 2: stained according to Lynch *et al.*[11]. Scale bar = 50 μm.
Fig. 3. Axonal labeling distal to the intranerval injection site. Survival time 6 days. Scale bar = 50 μm.
Fig. 4. HRP-positive fiber in one of the jaw muscles after intranerval injection. Scale bar = 50 μm.
Fig. 5. Cells in the trigeminal ganglion after peripheral intranerval HRP injection. Survival time 11 days. Scale bar = 25 μm.
Fig. 6. Labeling of fibers and cell bodies in the trigeminal ganglion after HRP injection in the trigeminal nerve 10 mm distal to the ganglion. Survival time 4 days. Scale bar = 25 μm.
Fig. 7. Cells in the trigeminal ganglion after peripheral intranerval HRP injection. Survival time 11 days. Scale bar = 25 μm.

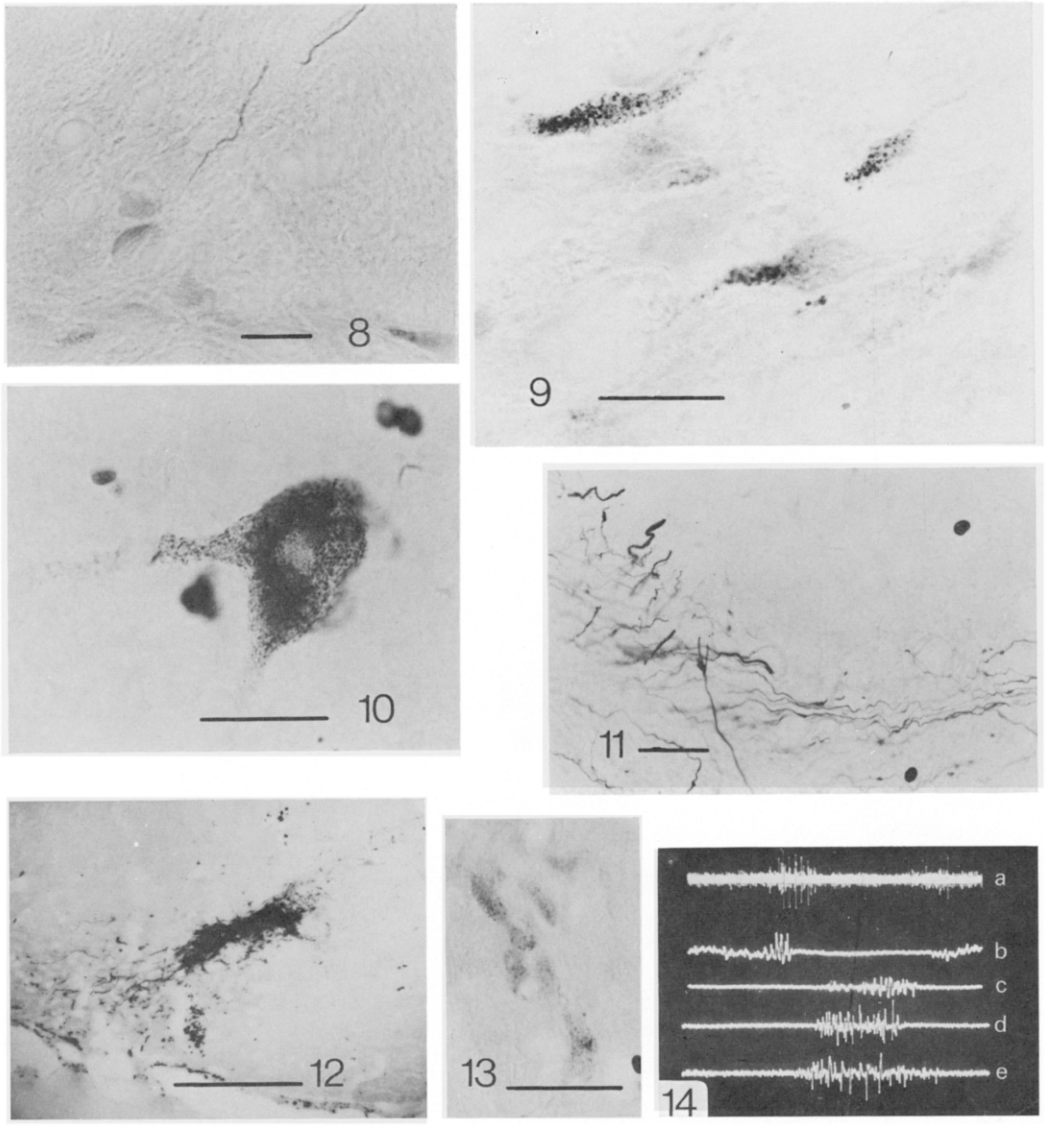


Fig. 8. Labeled axons reaching weakly labeled motor cell bodies after injection of HRP into a peripheral nerve. Survival time 6 days. Scale bar = 50 μm.

Figs. 9 and 10. HRP-positive cell bodies in the trigeminal motor nucleus following HRP injection in one of the jaw muscles. Survival time 11 days. Scale bar = 50 μm.

Fig. 11. Axonal labeling after electrophoretic HRP deposit in the cerebellum. Survival time 5 days. Scale bar = 50 μm.

Fig. 12. Site of electrophoretic HRP deposit in the trigeminal motor nucleus. Note that the enzyme is distributed in conformity with the shape of the nucleus. Delivery current: 2 μA for total on-time of 10 min. Scale bar = 500 μm.

Fig. 13. HRP-positive cells in dorsal parts of the mesencephalon in a case of HRP injection in the cerebellum. Survival time 10 days. Scale bar = 50 μm.

Fig. 14. a: extracellular recording by means of a glass micropipette filled with Tris–HCl 0.05 *M* and 5% HRP. Note the favorable signal-to-noise ratio. b–e: electromyograms of the 4 main respiratory muscles.

12 days a larger number of labeled cells were usually found in both ganglia and motor nuclei (Figs. 5, 7 and 8); the cell labeling in such cases tended to be more intensive, whereas axonal labeling had markedly decreased and was completely absent after survival times of more than 8 days. Distal to the injection sites HRP reaction product was found in nerve trunks (Fig. 3) and in terminal parts of fibers innervating the jaw muscles (Fig. 4). The HRP technique is currently used in this laboratory in an anatomical study of muscle innervation patterns.

The limited knowledge of the neuroanatomy of the carp brain, when *injecting in the CNS*, makes it difficult to find a distinct structure of which the connections are so well known that it can serve as a model for testing the usefulness of the HRP technique (groups 3, 4 and 5). None the less, the findings reported here appeared promising enough to justify the application of the method to problems of central nervous circuitry. The results of such experimental studies will be presented in detail in subsequent papers.

Mechanical injection of the tracer is impracticable in the fish brain because of the small size of the brain and the consequent difficulty of obtaining adequate localization of the enzyme. Electrophoresis, on the other hand, has proved to be an excellent technique for localized and reproducible HRP injection (Fig. 12); furthermore, the glass micropipettes at the same time can be used for electrophysiological recording (Fig. 14). The injection sites in the cases collected thus far measured about 400 μm in diameter. Slight damage to the tissue appeared to occur, permitting HRP to penetrate fibers of passage. This might be reduced by decreasing the current.

In all cases both anterograde and retrograde axonal transport could be observed, permitting the conclusion that HRP uptake occurs at terminal parts of the axons as well as at cell bodies or at the part of the cell where the axon arises.

Two days after HRP injection of the cerebellum only a small number of fibers had become labeled over a short distance. After 5 days, axons were labeled over distances of 5 mm, both in the anterograde (*e.g.* outgoing motor fibers in cases of HRP injection in the trigeminal motor nucleus) and in the retrograde direction (fibers in the mesencephalic tegmentum labeled by HRP injection in the cerebellum; Fig. 11). Furthermore, cell bodies close to the injection site were found labeled, *e.g.*, in the acoustical area following HRP deposit in the ventral cerebellum, or cells in the reticular formation after injection in the adjacent motor trigeminal nucleus. At longer survival times more remote cell bodies became labeled, *e.g.*, 10 days after HRP injection in the granular layer of the corpus cerebelli many small HRP-positive cells appeared in the mesencephalic tegmentum (Fig. 13).

The lower speed of the intra-axonal transport process in the CNS as compared to its rate in the peripheral nervous system was striking. Moreover, the present findings show that HRP labeling by intracellular transport takes considerably more time in teleosts than it does in higher vertebrates. All things considered, however, the method is less time-consuming than degeneration techniques, and it does not demand any special skill. The histological procedures are very simple to perform and highly reproducible. A comparison of Karnovsky-stained[2] sections with sections processed

according to the technique described by Lynch *et al.*[11] demonstrated a greater sensitivity of the Karnovsky method.

A comparison between HRP and degeneration techniques shows that the unavoidable damage along the insertion track in the former has no consequence, provided no HRP is lost in the electrode tracks, while in the latter it results in fiber degeneration often severely complicating the interpretation of the results. The HRP technique, moreover, permits demonstration of both afferent and efferent connections, although it has not yet been established that HRP labels all fibers ending in or orginating at the injection site. Besides, it has not been possible to determine with certainty whether HRP reaches the axonal terminals.

All things considered, it can be concluded that the HRP method is more convenient than the degeneration technique and offers additional possibilities.

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